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ORIGINAL TRANSLATIONAL SCIENCE

Interplay of sex hormones and long-term right ventricular adaptation in a Dutch PAH-cohort

Jessie van Wezenbeek, MSc,[a] Joanne A. Groeneveldt, MD,[a] Aida Llucià-Valldeperas, PhD,[a] Cathelijne E. van der Bruggen, MD, PhD,[a] Samara M.A. Jansen, MD,[a] A. Josien Smits, MD,[a] Rowan Smal, MSc,[a] Joost W. van Leeuwen, MSc,[a] Cris dos Remedios, PhD,[b,c] Anne Keogh, MD,[d,e] Marc Humbert, MD, PhD,[f,g,h] Peter Dorfmüller, MD, PhD,[f,g,h] Olaf Mercier, MD, PhD,[f,g,i] Christophe Guignabert, MD, PhD,[f,g] Hans W.M. Niessen, MD, PhD,[j] M. Louis Handoko, MD, PhD,[k] J. Tim Marcus, PhD,[l] Lilian J. Meijboom, MD, PhD,[l] Frank P.T. Oosterveer, BSc,[a] Berend E. Westerhof, PhD,[a,m] Annemieke C. Heijboer, MD, PhD,[n] Harm Jan Bogaard, MD, PhD,[a] Anton Vonk Noordegraaf, MD, PhD,[a] Marie José Goumans, PhD,[o] and Frances S. de Man, PhD[a]

From the [a]Department of Pulmonary Medicine, Amsterdam Cardiovascular Sciences, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands; [b]Victor Chang Cardiac Research Institute, Darlinghurst, Australia; [c]University of Sydney, Sydney, Australia; [d]Heart Transplant Unit, St Vincent's Public Hospital, Sydney, Australia; [e]University of NSW, Sydney, Australia; [f]INSERM UMR_S 999, Hypertension pulmonaire: physiopathologie et innovation thérapeutique, Le Plessis Robinson, France; [g]Université Paris-Saclay, Hypertension pulmonaire: physiopathologie et innovation thérapeutique, Le Plessis Robinson, France; [h]Assistance Publique - Hôpitaux de Paris (AP-HP), Department of Respiratory and Intensive Care Medicine, Pulmonary Hypertension National Referral Center, Hôpital Bicêtre, Le Kremlin-Bicêtre, France; [i]Department of Thoracic and Vascular Surgery and Heart-Lung Transplantation, Hôpital Marie Lannelongue, Le Plessis Robinson, France; [j]Department of Pathology, Amsterdam University Medical Centers, Amsterdam Cardiovascular Sciences, Amsterdam, The Netherlands; [k]Department of Cardiology, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam Cardiovascular Sciences, Amsterdam, The Netherlands; [l]Department of Radiology and Nuclear Medicine, Amsterdam UMC, Vrije Universiteit, Amsterdam Cardiovascular Sciences, Amsterdam, The Netherlands; [m]University of Twente, Technical Medical Centre, Cardiovascular and Respiratory Physiology, Enschede, The Netherlands; [n]Endocrine Laboratory, Department of Clinical Chemistry, Amsterdam UMC, Vrije Universiteit Amsterdam and University of Amsterdam, Amsterdam Gastroenterology Endocrinology & Metabolism, Amsterdam, The Netherlands; and the [o]Department of Cell and Chemical Biology, Leiden UMC, Leiden, the Netherlands.

BACKGROUND: To investigate the association between altered sex hormone expression and long-term right ventricular (RV) adaptation and progression of right heart failure in a Dutch cohort of Pulmonary Arterial Hypertension (PAH)-patients across a wide range of ages.

Reprint requests: Frances S. de Man, PhD, Department of Pulmonary Medicine, Amsterdam Cardiovascular Sciences, Amsterdam UMC, Vrije Universiteit Amsterdam, PHEniX Laboratory, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands. Telephone: +31-20 444 1883.
E-mail address: fs.deman@amsterdamumc.nl

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METHODS: In this study we included 279 PAH-patients, of which 169 females and 110 males. From 59 patients and 21 controls we collected plasma samples for sex hormone analysis. Right heart catheterization (RHC) and/or cardiac magnetic resonance (CMR) imaging was performed at baseline. For longitudinal data analysis, we selected patients that underwent a RHC and/or CMR maximally 1.5 years prior to an event (death or transplantation, N = 49).

RESULTS: Dehydroepiandrosterone-sulfate (DHEA-S) levels were reduced in male and female PAH-patients compared to controls, whereas androstenedione and testosterone were only reduced in female patients. Interestingly, low DHEA-S and high testosterone levels were correlated to worse RV function in male patients only. Subsequently, we analyzed prognosis and RV adaptation in females stratified by age. Females ≤45years had best prognosis in comparison to females ≥55years and males. No differences in RV function at baseline were observed, despite higher pressure-overload in females ≤45years. Longitudinal data demonstrated a clear distinction in RV adaptation. Although females ≤45years had an event at a later time point, RV function was more impaired at end-stage disease.

CONCLUSIONS: Sex hormones are differently associated with RV function in male and female PAH-patients. DHEA-S appeared to be lower in male and female PAH-patients. Females ≤45years could persevere pressure-overload for a longer time, but had a more severe RV phenotype at end-stage disease.

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Pulmonary arterial hypertension (PAH) is a progressive disease characterized by narrowing of pulmonary arterioles and right heart failure.[1-3] Although females are more prone to develop PAH (female: male prevalence ~2:1), their survival is significantly better than male patients.[4-8] Previous studies showed that this survival benefit in females may be explained by a different RV response to treatment.[4,9]

Sex differences are common in cardiovascular physiology and disease. The multi-ethnic study of atherosclerosis (MESA) in 4000 healthy community-based participants revealed that RV mass and volume are smaller in females, while RV ejection fraction (RVEF) is higher compared to age-matched males.[10] To a great extent, this distinction was explained by differences in sex hormone levels, especially estrogens.[11] Higher RVEF and lower RV end-systolic volume were associated with higher estrogen serum levels in healthy postmenopausal women using hormone replacement therapy (HRT).[11]

Different expression levels of sex hormones have also been observed in PAH.[12-17] Especially high estradiol and reduced dehydroepiandrosterone-sulfate (DHEA-S) have been associated with PAH-severity in male and postmenopausal females. In addition, several animal studies have investigated the effect of sex hormones on pulmonary vascular remodeling and RV failure.[12] However, due to conflicting data between patients and animal models, and due to limited data in younger females of reproductive age, the exact association between sex hormones and RV adaptation remains elusive. In addition, most data on sex hormones has been obtained in US centers. With the known differences in demographic characteristics and HRT use between US and European patients, it is of importance to investigate sex hormones in a non-US PAH-cohort as well.[18] Finally, the majority of data is cross-sectional or at one point in time during the disease course, whereas longitudinal data of RV function during progression towards right heart failure is pivotal to understand differential RV adaptation patterns between male and female patients. Therefore, in this study, we aimed to investigate the association between altered sex hormone expression levels and RV adaptation and to assess progression of right heart failure over time in a Dutch cohort of patients with a wide range of ages. We performed a translational study in which we combined cross-sectional analyses of sex hormones with longitudinal analyses of RV adaptation and histopathological analyses of RV tissue from female and male PAH-patients.

Materials and methods

More detailed information in the supplementary material.

Study design and patients for RV function assessment

We screened N = 305 PAH-patients for inclusion that were diagnosed according to European Respiratory Society (ERS) and European Society of Cardiology (ESC) guidelines at Amsterdam UMC (location VUmc) between March 1996 and September 2019. At baseline, patients underwent right heart catheterization (RHC) and/or cardiac magnetic resonance (CMR) imaging within a maximum interval of one month, prior to receiving PAH-specific therapy. We cross-sectionally collected plasma samples for sex hormone analysis from 59 patients and 21 controls at the clinical visit including a RHC and/or CMR. (Medical Ethics Approval Numbers 2015.220-Liquid Biopsy and 2017.318-DOLPHIN). PAH-patients included both treatment naïve (N = 15) and treated (N=44) patients. Control subjects included subjects that were seen at our hospital for evaluation of dyspnea or had a positive mutation carrier status or where family members of a patient had a positive mutation carrier status, but had normal pulmonary artery pressures. None of these controls had any cardiovascular or pulmonary disease.

Furthermore, as estrogen levels are highly variable during the menstrual cycle, and since estrogen levels drop around the age of 50 years, we were unable to measure estradiol levels in females. Therefore, we stratified the female cohort on age. We included 279 subjects in this sub-analysis to study associations between estrogens and RV adaptation: females of reproductive age (N = 72, ≤45 years), females of postmenopausal age (N = 97, ≥55 years old), and males (N = 110). In addition, we studied differences in survival and differences in end-stage disease. Survival was defined as the time from the baseline measurements prior to initiation of treatment to a first event (death or transplantation) or to the censoring date. Of 49 patients that had an event (death or transplantation), a last RHC and/or CMR measurement was available with a maximum of 1.5 years before the event. From these patients we collected RHC and CMR data at earlier time points during the disease. Lastly, in explanted and post-mortem RV tissue of male and female patients (N = 6) and controls (N = 6), we assessed histopathological differences. The Medical Ethics Review Committee of Amsterdam UMC did not consider the current study to fall within the scope of the Medical Research Involving Human Subjects (WMO), therefore informed consent was not required (approval number 2012288). Approval from the Sydney Heart Bank was received for use of RV samples (HREC #2814; HREC #7326). Use of human RV autopsy samples was approved by and performed according to guidelines of the ethics committee of Amsterdam UMC, location VUmc, and conformed to the Declaration of Helsinki principles.

Sex hormone analysis

All samples were analyzed at the Endocrine Laboratory of Amsterdam UMC. DHEA-S, testosterone, androstenedione, and estradiol (in males only) were measured in plasma using isotope diluted liquid chromatography-mass spectrometry (ID-LC-MS/MS).[19-21] Sex hormone-binding globulin (SHBG) was measured using an automated immunoassay (Architect, Abbott Diagnostics). Free testosterone levels were calculated using Vermeulen.[22]

Right heart catheterization

Hemodynamic assessment was performed using a balloon-tipped, flow-directed 7.5-F triple lumen Swan-Ganz catheter (Edwards Lifesciences LLC, Irvine, CA, USA), as previously described.[23] RV arterial coupling analysis was performed as previously described [23] to obtain the end-systolic elastance (Ees) as measure for RV contractility, arterial elastance (Ea) as measure for RV afterload and the end-diastolic elastance (Eed) as measure for RV diastolic stiffness.

Cardiac magnetic resonance imaging

Measures of RV function and volumes were taken using CMR imaging. Scans were obtained using a Siemens 1.5-T Sonata or Avanto scanner (Siemens Medical Solutions, Erlangen, Germany). Acquisition of images and post-processing was carried out as reported.[24]

Immunohistofluorescence

Right ventricle tissue from deceased healthy subjects (N = 3 females, N = 3 males) and both deceased or transplanted PAH-patients (N = 3 females, N = 3 males) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 24 hours, and embedded into paraffin. Afterwards, the paraffin block was cut into 5-μm sections and mounted into the slide with 3-aminopropyl-triethoxysilane (APES).

Tissue sections were dewaxed with two 3-min xylene washes, one 3-min xylene:ethanol, 3-min graded ethanol washes of 100% (twice), 95%, 70%, and 50% followed by running cold tap water. Next, sections were steamed in antigen retrieval citrate-based pH=6 solution for 30 min at high pressure. The sections were permeabilized, blocked, and incubated at 4˚C overnight with the primary antibody. Sections were stained for cluster of differentiation 31 (also known as *Platelet endothelial cell adhesion molecule* (PECAM-1)) (CD31, diluted 1:50; Santa Cruz Biotechnologies). Samples were then incubated for 1 hour at room temperature with anti-mouse secondary antibody conjugated with Alexa-488 (diluted 1:500; Abcam). The sections were counterstained with Hoechst 33342 nuclear dye (diluted 1:500; Santa Cruz Biotechnology), rhodamine Ulex europaeus agglutinin I (Ulex, diluted 1:200; Vectorlabs), and Alexa-647 conjugated wheat germ agglutinin (WGA, diluted 1:300; Thermo Fisher Scientific). Finally, samples were coverslipped with ProLong Gold Antifade Mountant (Thermofisher Scientific). Cell apoptosis was assessed by Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay using the *In situ cell death detection kit fluorescein* (Roche) following manufacturer's protocol. Images were captured at 20x, 40x and 60x magnifications under a laser confocal microscope (Nikon A1R). Whole-slide image acquisition was performed on Vectra Polaris (Akoya) at 10x and 20x magnifications. Quantitative histological measurements (cardiomyocyte cross-sectional area, vessel density and apoptotic nuclei) were assessed through ImageJ analysis software (NIH) with at least 10 random fields averaged per patient.

PECAM-1 Antibody (C-20) (#sc-1505, Santa Cruz Biotechnologies)	1:50
Wheat Germ Agglutinin (WGA), Alexa Fluor 647 Conjugate (#W32466, Thermofisher)	1:500
Lectin-rhodamine "Ulex Europaeus Agglutinin I" (Ulex, #RL1062, Vectorlabs)	1:200
Hoechst 33342 trihydrochloride (CAS 23491-52-3, #sc-200908, Santa Cruz Biotechnologies)	1:500
In situ cell death detection kit fluorescein (#11684795910, Roche)	

Statistical analysis

Data are presented as mean (standard deviation) or median [interquartile range]. Transplant-free differences in survival were assessed using Kaplan-Meier curves and log-rank test with Bonferroni-corrected pairwise comparison. We corrected the survival analysis for confounders including body mass index (BMI), treatment and comorbidities using cox regression analysis. For normally distributed continuous variables, group differences were assessed using one-way ANOVA and post-hoc unpaired t-tests with Bonferroni correction. For non-normally distributed data, logarithmic transformation was applied prior to testing or

Kruskal-Wallis test and post-hoc testing with pairwise Mann −Whitney U-tests was performed. For categorical variables, group differences were assessed with Pearson's $\chi 2$ test or Fisher's exact test. A repeated measures ANOVA was used to assess differences in change of variables over time between groups, after which post-hoc testing was performed using a t-test with Bonferroni correction to assess differences within and between groups. Univariate and multivariate regression was used to assess the relation between two or more continuous variables and to check and if needed correct for potential confounders. Differences between groups were assessed using interaction testing included in the regression model. A p-value of < 0.05 was considered statistically significant. Statistical analyses were performed in R (version 3.5.2).

Results

PAH-induced sex differences in survival, RV function and adaptation

We included 236 idiopathic PAH, 33 hereditable PAH, 9 drug- and toxin-induced PAH, and 1 PAH patient associated with HIV-infection, resulting in 110 male and 169 female patients in total in the age-stratified PAH cohort (N = 279) (Figure 1).

Low DHEA-S and high testosterone levels associated with worse RV function in male PAH

To investigate the association of RV adaptation and sex hormones, we quantified plasma levels of sex hormones in a subgroup of patients. We cross-sectionally determined levels of precursors to estrogens and androgens (Figure S1) in 23 male and 36 female PAH-patients, and 11 male and 10 female healthy controls. No differences were observed in therapy or medication type at time of blood sampling between male and female PAH-patients.

Characteristics of the cross-sectional cohort are presented in Table 1 and compared to the main baseline cohort in Table S1. Disease severity was worse in the baseline cohort which included only treatment naïve patients, as shown by a slightly worse RV function and worse functional class compared to the cross-sectional cohort, which included both treatment naïve and treated patients.

Levels of DHEA-S were reduced in both female and male PAH-patients compared to control subjects (Figure 2A). Intriguingly, androstenedione levels, as well as total and free testosterone levels, were significantly lower in female patients compared to controls, but did not differ between male patients and controls (Figure 2B-D). In addition, although estradiol levels appeared slightly higher in PAH males, levels were not significantly increased compared to controls (Figure S2).

Next, we investigated the association between sex hormone levels and RV functional and PH disease parameters. Levels of DHEA-S were negatively associated with RV afterload, RV contractility as well as RV diastolic stiffness in both sexes (Figure 3A, B and Figure S2). Interestingly, the relationship between DHEA-S with RVEF was different for male and female patients: lower DHEA-S levels were correlated to worse RVEF in males, whereas no correlations

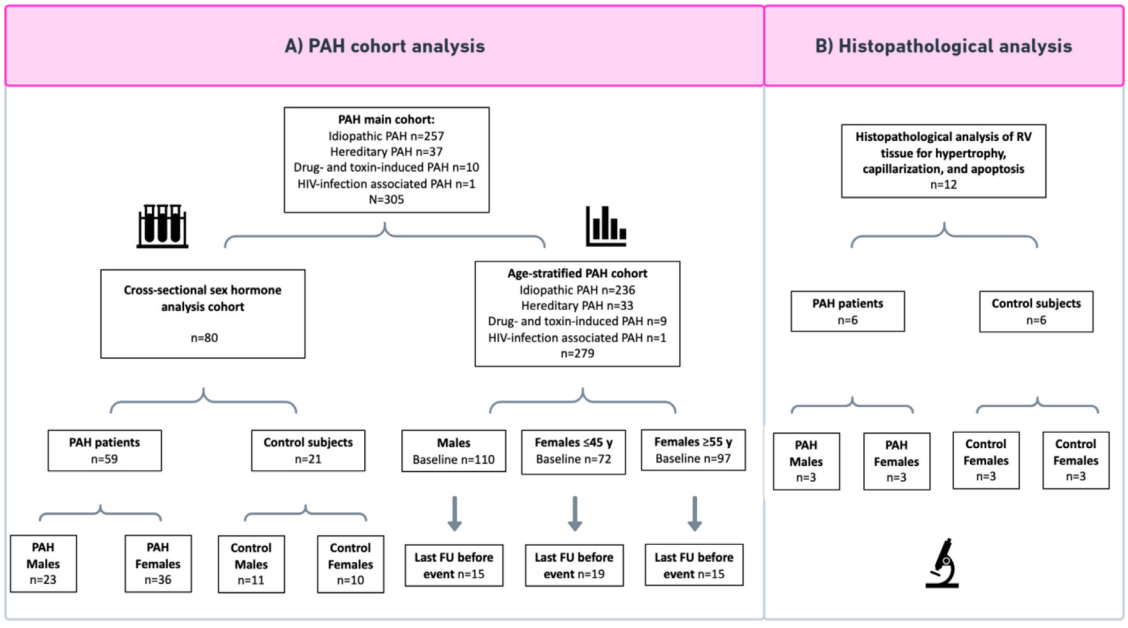


Figure 1 Flow chart schematic overview of study populations used for each analysis. At baseline, we screened N = 305 PAH-patients for inclusion. We cross-sectionally collected plasma samples for sex hormone analysis from 59 patients and 21 controls. Furthermore, we performed a sub-analysis in the female cohort stratified on age. We included 279 subjects in the age-stratified PAH cohort: females of reproductive age (N = 72, ≤45 years), females of postmenopausal age (N = 97, ≥55 years old), and males (N = 110). In addition, we studied differences in end-stage disease. Of 49 patients that had an event (death or transplantation), a last RHC and/or CMR measurement was available with a maximum of 1.5 years before the event. Lastly, we studied histopathological differences in explanted and post-mortem RV tissue of male and female patients (N = 6) and controls (N = 6). F, Female; F ≤45, Females ≤45 years; F ≥55, Females ≥55 years; HIV, Human Immunodeficiency Virus; M, Male; PAH, Pulmonary Arterial Hypertension; RV, Right Ventricular.

Table 1 Characteristics of the Subgroup of Patients and Controls in the Sex Hormone Analysis

Characteristic	Control F	PAH F	Control M	PAH M	*p*-value
Number	10	36	11	23	
Age at visit, years	48 (20)	51 (16)	46 (19)	50 (14)	0.99
BSA, m^2	1.7 (0.1)	1.8 (0.2) [a]	2.0 (0.2)	2.0 (0.2)	<0.001
BMI, kg/m^2	24 (3)	26 (5)	25 (4)	25 (4)	0.74
NYHA FC					
Class I, *n* (%)		3 (8)		2 (9)	
Class II, *n* (%)		25 (69)		16 (70)	
Class III, *n* (%)		8 (22)		5 (21)	0.99
NTproBNP, ng/l		202 [111 - 399]		172 [105 - 821]	0.64
RHC					
mPAP, mmHg		48 (14)		47 (13)	0.77
mRAP, mmHg		7 [6 - 9]		7 [5 - 10]	0.67
PAWP, mmHg		12 (3)		11 (3)	0.46
PVR, WU		7 [4 - 11]		7 [4 - 8]	0.22
Cardiac index, l/m^2		2.9 (0.7)		3.1 (0.9)	0.43
CMR					
Heart rate, bpm	72 (9)	72 (12)	67 (17)	71 (13)	0.74
RVEDV index, ml/m^2	66 (17)	89 (27) [b]	83 (20)	98 (20)	<0.01
RVESV index, ml/m^2	30 (14)	48 (22)	37 (13)	60 (23) [c]	<0.01
SV index, ml/m^2	40 (6)	40 (11)	52 (11)	41 (11)	0.90
RVEF, %	59 (6)	48 (11)[a]	56 (8)	40 (14)	<0.001
RV mass index, g/m^2	25 (2)	43 (14)[b]	27 (6)	53 (17)[c]	<0.001
Therapy					
Monotherapy, *n* (%)		11 (31)		8 (35)	
Dual therapy, *n* (%)		19 (53)		11 (48)	
Triple Therapy, *n* (%)		6 (17)		4 (17)	0.93
Medication type					
ERA, *n* (%)		25 (69)		21 (91)	0.10
PDE5, *n* (%)		29 (81)		15 (65)	0.31
Prostacyclin, *n* (%)		12 (33)		4 (20)	0.45
Hormonal treatment					
Contraceptive use, *n* (%)	1 (10)	6 (17)			
Hormone replacement therapy, *n* (%)	0 (0)	1 (3)			

BMI, Body Mass Index; BPM, Beats Per Minute; BSA, Body Surface Area; CMR, Cardiac Magnetic Resonance; ERA, Endothelin Receptor Antagonist; mPAP, mean Pulmonary Arterial Pressure; mRAP, mean Right Atrial Pressure; NYHA FC, New York Heart Association Functional Class; PAWP, Pulmonary Artery Wedge Pressure; PDE5, Phosphodiesterase type 5 inhibitor; PVR, Pulmonary Vascular Resistance; RVEDV, Right Ventricular End-Diastolic Volume; RVESV, Right Ventricular End-Systolic Volume; RVEF, Right Ventricular Ejection Fraction; RHC, Right Heart Catheterization; SV, Stroke Volume.

Data is presented as mean (SD) or as median [IQR] or as *n* (%)

[a]Male vs female PAH-patients

[b]Female PAH-patients vs Female controls

[c]Male PAH-patients vs Male controls.

were observed in females (Figure 3C, D). In both male and female patients, lower levels of DHEA-S were related to higher levels of NTproBNP (Figure 3E). Interestingly, we observed a positive correlation between estradiol levels and PAP and right atrial pressure, and we observed a negative correlation between testosterone levels and RVEF in male PAH-patients only (Figure 3F; Figure S2). These data indicate that the association between DHEA-S and testosterone with RV function different in male and female patients.

Superior RV adaptation in females of reproductive age

Several indications in literature point to the direction of estrogen as main regulator of RV adaptation in PAH. Although we did not observe any relation between estradiol and RV functional parameters in male patients, our results indicate that high estradiol levels were associated with more pronounced disease severity (Figure S2). Since estrogen levels are variable throughout the menstrual cycle, it is challenging to interpret estrogen plasma levels in females.[17] As estrogen levels are known to drop around the age of 50 years,[25] to get a general idea on the contribution of estrogens to RV adaptation in female patients, we stratified the female cohort on age: females of reproductive age (N = 72, ≤45 years), and females of postmenopausal age (N = 97, ≥55 years old). The male cohort was included as a reference group and was not stratified on age. Baseline characteristics are presented in Table 2. Females ≤45years had a higher BMI and *BMPR2* mutations were more prevalent. A low diffusing capacity of the lung for carbon monoxide

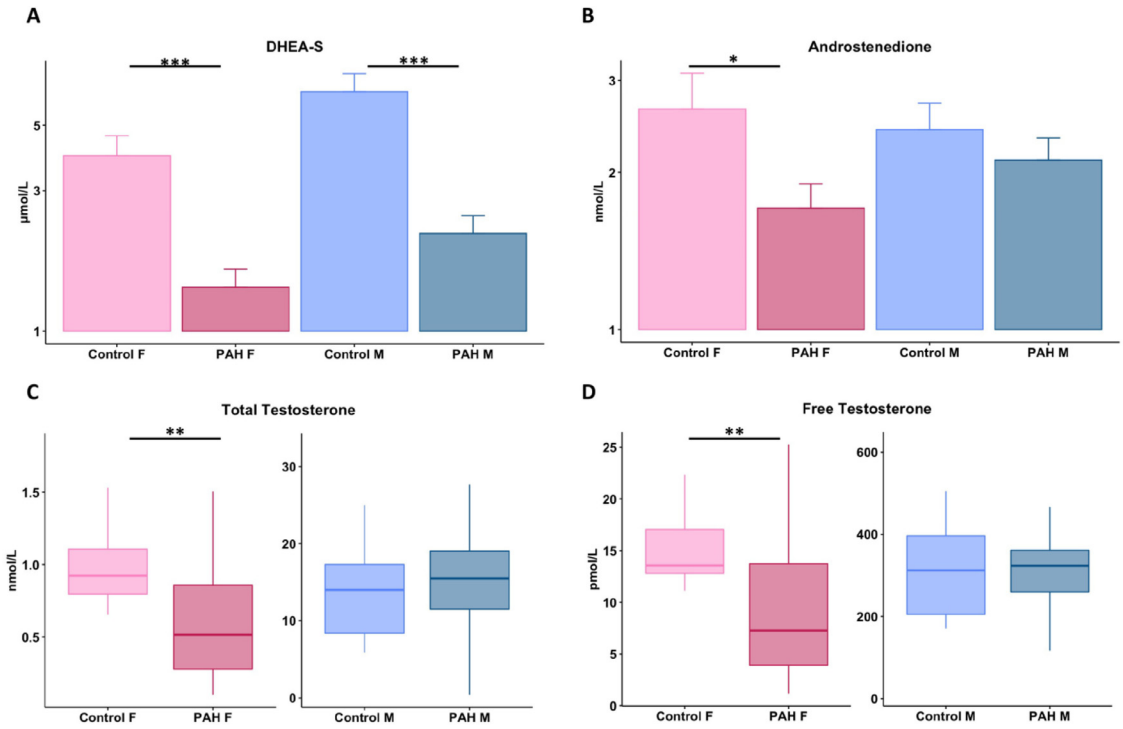


Figure 2 **Lower DHEA-S and lower testosterone in PAH-patients compared to controls.** Cross-sectional hormone analysis of male (N = 23) and female PAH-patients (N = 36) and male (N = 11) and female controls (N = 10) for DHEA-S (A) Androstenedione (B) Total testosterone (C) and Calculated free testosterone (D) levels. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. DHEA-S, Dehydroepiandrosterone-sulfate; F, Females; M = Males; PAH, Pulmonary Arterial Hypertension.

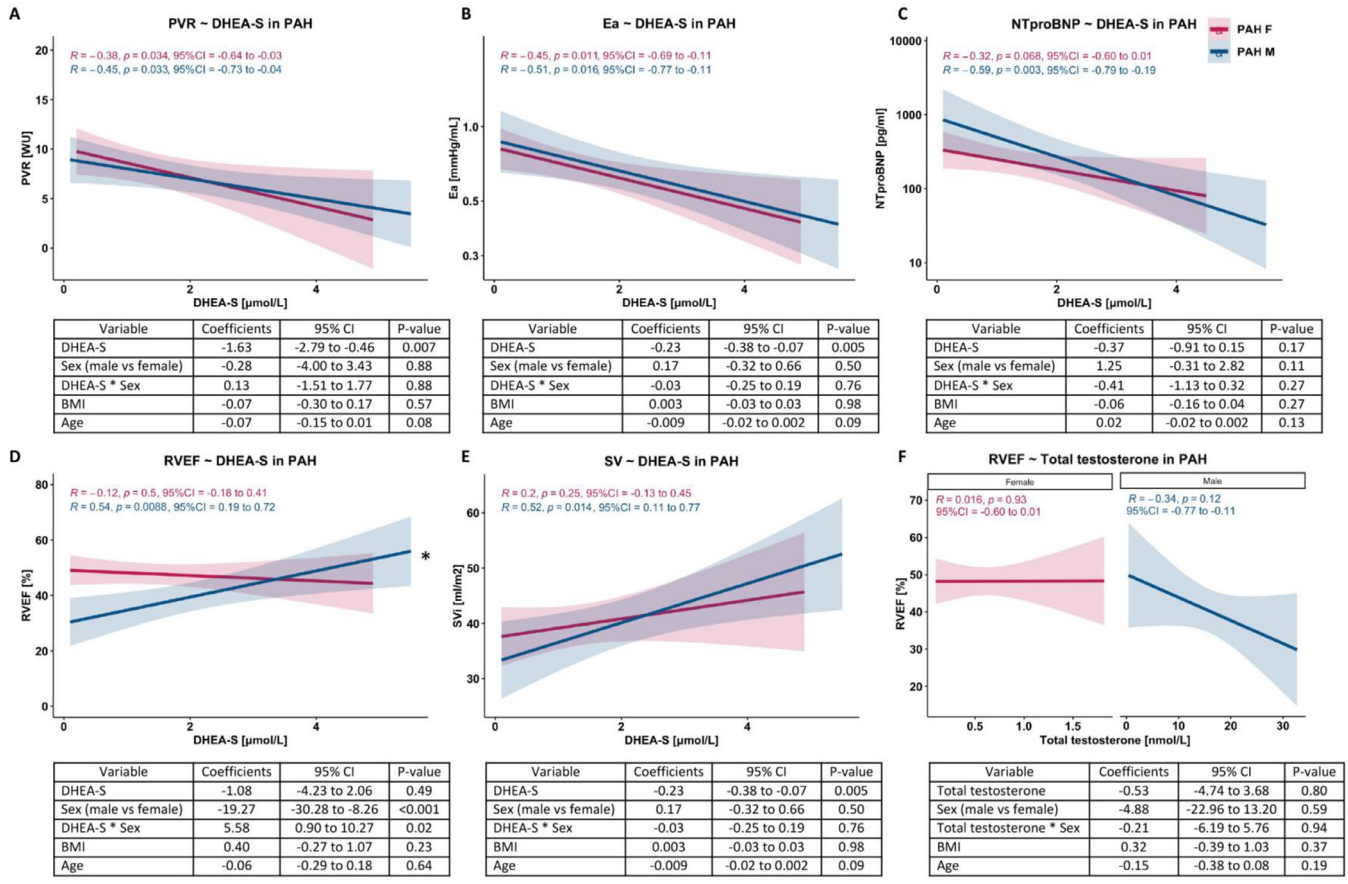
(DLCO) (≤45%) and comorbidities were more prevalent in male PAH-patients. The frequency distribution of mono-, duo- and triple therapy was comparable between groups, although PDE5 inhibitors were more frequently provided to males and females ≥55years and prostacyclin therapy more frequently to females ≤45years.

With the female cohort stratified on age, females ≤45years had the best prognosis, whereas males had the worst prognosis. These differences remained after correction for BMI, comorbidities, and differences in treatment (Figure 4A, Table S2). Little differences in hemodynamics and RV functional data could be observed at baseline between females ≤45years, females ≥55years, and males (Figure 4, Figure S3). Only RV afterload (mPAP, PVR, and Ea) was significantly higher in females ≤45years in comparison to females ≥55years and males (Figure 4B, Figure S3, Table 2). These data suggest a superior adaptation in females ≤45years, as they have a similar RVEF with a higher RV afterload, also reflected by the difference in RVEF ~ mPAP slope (Figure 4F).

Next, to investigate longitudinal differences in RV adaptation over time, we selected all patients with an event (defined as death or transplantation) and a CMR and/or RHC measurement max. 1.5 years prior to the event (Table S3). In addition, we analyzed CMR and/or RHC data at three earlier time points during the disease including baseline measurements. Median time from the last CMR and/or RHC measurement to the event was similar for all patients (females ≤45years: 0.47 vs females ≥55years: 0.77 vs males: 0.52 years, $p = 0.42$), whereas median time from baseline to the last CMR and/or RHC measurement to the event was significantly longer in females of reproductive age, as expected from the survival curve (females ≤45years: 5.2 vs females ≥55years: 2.0 vs males: 1.8 years, respectively, $p < 0.05$). Intriguingly, although no differences could be observed at baseline (Table S3), longitudinal analyses revealed significant altered response to pressure-overload over time between females ≤45years, females ≥55years, and males (Figure 5B-D). In contrast to females ≤45years and males, no signs of RV dilatation or RV dysfunction are observed in females ≥55years. At an earlier point in time males show RV dilatation which coincides with a drop in RVEF. Remarkably, although females ≤45years have a higher mPAP during the whole follow-up period, they persevere RV afterload the best and only show severe signs of RV dilatation and reduced RVEF at late end-stage disease. Note that the extent of RV dilatation and RV dysfunction is most pronounced in females ≤45years suggesting a more severe RV phenotype at end-stage disease.

Sex differences in end-stage RV tissue

To determine whether we could confirm a more severe RV phenotype in female PAH-patients at end-stage disease, we collected post-mortem and transplanted RV tissue. RV hypertrophy, capillary density, and apoptosis were assessed in RV tissue of end-stage male and female PAH-patients and were compared to controls (Figure 6). Female tissue was obtained of PAH-patients' with an age <55 years old, while control tissue of female healthy subjects was



Variable	Coefficients	95% CI	P-value
DHEA-S	-1.63	-2.79 to -0.46	0.007
Sex (male vs female)	-0.28	-4.00 to 3.43	0.88
DHEA-S * Sex	0.13	-1.51 to 1.77	0.88
BMI	-0.07	-0.30 to 0.17	0.57
Age	-0.07	-0.15 to 0.01	0.08

Variable	Coefficients	95% CI	P-value
DHEA-S	-0.23	-0.38 to -0.07	0.005
Sex (male vs female)	0.17	-0.32 to 0.66	0.50
DHEA-S * Sex	-0.03	-0.25 to 0.19	0.76
BMI	0.003	-0.03 to 0.03	0.98
Age	-0.009	-0.02 to 0.002	0.09

Variable	Coefficients	95% CI	P-value
DHEA-S	-0.37	-0.91 to 0.15	0.17
Sex (male vs female)	1.25	-0.31 to 2.82	0.11
DHEA-S * Sex	-0.41	-1.13 to 0.32	0.27
BMI	-0.06	-0.16 to 0.04	0.27
Age	0.02	-0.02 to 0.002	0.13

Variable	Coefficients	95% CI	P-value
DHEA-S	-1.08	-4.23 to 2.06	0.49
Sex (male vs female)	-19.27	-30.28 to -8.26	<0.001
DHEA-S * Sex	5.58	0.90 to 10.27	0.02
BMI	0.40	-0.27 to 1.07	0.23
Age	-0.06	-0.29 to 0.18	0.64

Variable	Coefficients	95% CI	P-value
DHEA-S	-0.23	-0.38 to -0.07	0.005
Sex (male vs female)	0.17	-0.32 to 0.66	0.50
DHEA-S * Sex	-0.03	-0.25 to 0.19	0.76
BMI	0.003	-0.03 to 0.03	0.98
Age	-0.009	-0.02 to 0.002	0.09

Variable	Coefficients	95% CI	P-value
Total testosterone	-0.53	-4.74 to 3.68	0.80
Sex (male vs female)	-4.88	-22.96 to 13.20	0.59
Total testosterone * Sex	-0.21	-6.19 to 5.76	0.94
BMI	0.32	-0.39 to 1.03	0.37
Age	-0.15	-0.38 to 0.08	0.19

Figure 3 Sex hormones are differently related to RV function in PAH males and females. Relationship between levels of DHEA-S and PVR in (A) DHEA-S and Ea or arterial elastance as measure for RV afterload in (B) DHEA-S and RVEF in (C) DHEA-S and SV in (D) DHEA-S and NTproBNP in (E) and total testosterone and RVEF (F) in male (N = 23) and female PAH-patients (N = 36). Differences between groups were tested using interaction testing as depicted by the interaction term in the table. DHEA-S, Dehydroepiandrosterone-sulfate; F, Females; M, Males; PAH, Pulmonary Arterial Hypertension; RVEF, Right Ventricular Ejection Fraction; SV, Stroke Volume.

>51 years old. Although no statistical analyses could be performed due to limited number of tissue samples per group, representative images suggest a more pronounced capillary rarefaction and vascular apoptosis in RV tissue of female patients in comparison to male patients, also indicating a more severe RV phenotype in females compared to male PAH-patients.

Discussion

Using a comprehensive set of hormone analyses combined with longitudinal imaging and hemodynamic data, we were able to demonstrate that:

1. For the first time, we show a differential association between sex hormones and RV function in male and female PAH-patients.
2. DHEA-S plasma levels are reduced in male and female PAH-patients across a wide range of ages, whereas reduced testosterone and androstenedione levels are observed in female patients only.
3. Females of reproductive age may tolerate RV pressure-overload for a longer period of time resulting in a more severe RV phenotype at end-stage disease.

Sex differences in association of DHEA-S with RV function

In healthy subjects DHEA-S levels were associated with larger RV volumes and mass but worse RV function in females, however not in males.[10] In contrast to observations in healthy subjects, DHEA-S levels were reduced in both postmenopausal female and older male PAH-patients, and throughout the menstrual cycle in a small study of reproductive PAH females.[15-17] Furthermore, DHEA-S levels were associated with disease severity, progression and survival.[15-17] In experimental studies, DHEA-S has shown to prevent pulmonary vascular changes and may thereby positively affect the right ventricle. DHEA-S treatment improved both systolic and diastolic RV function, and reduced RV maladaptive remodeling by reversing cardiomyocyte hypertrophy and inhibiting RV capillary rarefaction and fibrosis.[26-28] In our study, we could confirm reduced expression of DHEA-S in a Dutch cohort of male and female PAH-patients across a wide range of ages. This further supports the rationale of a phase 2 clinical trial currently conducted to study effects of DHEA supplementation on RV functional parameters

Table 2 Baseline Characteristics of Female PAH-Patients Stratified on Age

Characteristic	Females ≤45 years	Females ≥55 years	Males	*p*-value
Number	72	97	110	
Age at Diagnosis, years	34 (8) [a,b]	68 (8)	61 (17)	<0.001
BSA, m^2	1.9 (0.3)	1.9 (0.2)[c]	2.0 (0.2)	<0.01
BMI, kg/m^2	28 (7)	28 (5) [c]	26 (4)	<0.01
sABP, mmHg	125 (16)	134 (25)	127 (20)	<0.05
dABP, mmHg	76 (12)	75 (15)	76 (14)	0.79
NYHA FC				
Class I, *n* (%)	5 (8)	1 (1)	3 (4)	
Class II, *n* (%)	17 (29)	21 (28)	27 (34)	
Class III, *n* (%)	29 (49)	42 (57)	39 (49)	
Class IV, *n* (%)	8 (14)	10 (14)	11 (14)	0.52
6MWD, m	448 [335-497] [a,b]	314 [218-415]	330 [186-456]	<0.001
NTproBNP, ng/l	813 [355-1932]	1269 [369-2945]	1090 [299-2870]	0.44
HPAH BMPR2, *n* (%)	15 (21) [a]	6 (6)	10 (9)	<0.05
Low DLCO, *n* (%)	0 (0) [b]	14 (14) [c]	35 (32)	<0.001
Comorbidities				
Smoking, n (%)	25 (36) [a,b]	46 (54)	64 (65)	<0.01
Coronary Artery Disease, *n (%)*	0 (0) [a]	13 (15) [c]	25 (25)	<0.001
Hypertension, *n* (%)	8 (12) [a]	37 (44)	30 (30)	<0.001
Diabetes, *n* (%)	3 (4) [a]	25 (29)	18 (18)	<0.001
RHC				
sPAP, mmHg	88 (23) [a,b]	80 (19)	78 (19)	<0.001
dPAP, mmHg	38 (13) [a,b]	31 (10)	32 (10)	<0.001
mPAP, mmHg	57 (15)[a,b]	49 (12)	49 (12)	<0.001
mRAP, mmHg	9 [5 - 11]	8 [6 - 11]	7 [5 - 11]	0.59
PAWP, mmHg	9 (3)	11 (3)[c]	9 (3)	0.001
PVR, WU	11 [7 - 14] [b]	9 [6 - 15]	8 [6 - 12]	<0.05
Cardiac index, l/m^2	2.5 (0.8)	2.5 (0.9)	2.4 (0.8)	0.88
CMR				
Heart rate, bpm	82 (14)	78 (10)	77 (15)	0.14
RVEDV index, ml/m^2	83 (21)	79 (30)	87 (23)	0.25
RVESV index, ml/m^2	56 (19)	51 (28)	61 (24)	0.13
SV index, ml/m^2	27 (8)	28 (8)	28 (8)	0.82
RVEF, %	33 (11)	37 (13)	33 (12)	0.10
RV mass index, g/m^2	53 (14)	47 (13)	56 (15)	<0.05
Therapy				
Monotherapy, *n* (%)	45 (63)	60 (62)	70 (64)	
Dual therapy, *n* (%)	23 (32)	33 (34)	32 (29)	
Triple therapy, *n* (%)	2 (3)	1 (1)	1 (1)	0.75
Medication type				
ERA, *n* (%)	42 (58)	63 (65)	59 (54)	0.25
PDE5, *n* (%)	28 (39)	56 (58)	59 (54)	<0.05
Prostacyclin, *n* (%)	21 (30) [a]	6 (6)	15 (14)	<0.05

6MWD, 6-min Walking Distance; BMI, Body Mass Index; BSA, Body Surface Area; CMR, Cardiac Magnetic Resonance; dABP, diastolic Arterial Blood Pressure; DLCO, Diffusion Capacity of the Lung for Carbon Monoxide; dPAP, diastolic Pulmonary Arterial Pressure; ERA, Endothelin Receptor Antagonist; HPAH, Hereditable Pulmonary Arterial Hypertension; mPAP, mean Pulmonary Arterial Pressure; mRAP, mean Right Atrial Pressure; NYHA FC, New York Heart Association Functional Class; PAWP, Pulmonary Artery Wedge Pressure; PDE5, Phosphodiesterase type 5 inhibitor; PVR, Pulmonary Vascular Resistance; RVEDV, Right Ventricular End-Diastolic Volume; RVESV, Right Ventricular End-Systolic Volume; RHC, Right Heart Catheterization; RVEF, Right Ventricular Ejection Fraction; sABP, systolic Arterial Blood Pressure; sPAP, systolic Pulmonary Arterial Pressure; SV, Stroke Volume.

Data are presented as mean (SD) or as median [IQR] or as *n* (%)

[a]Females ≤45 vs females ≥55

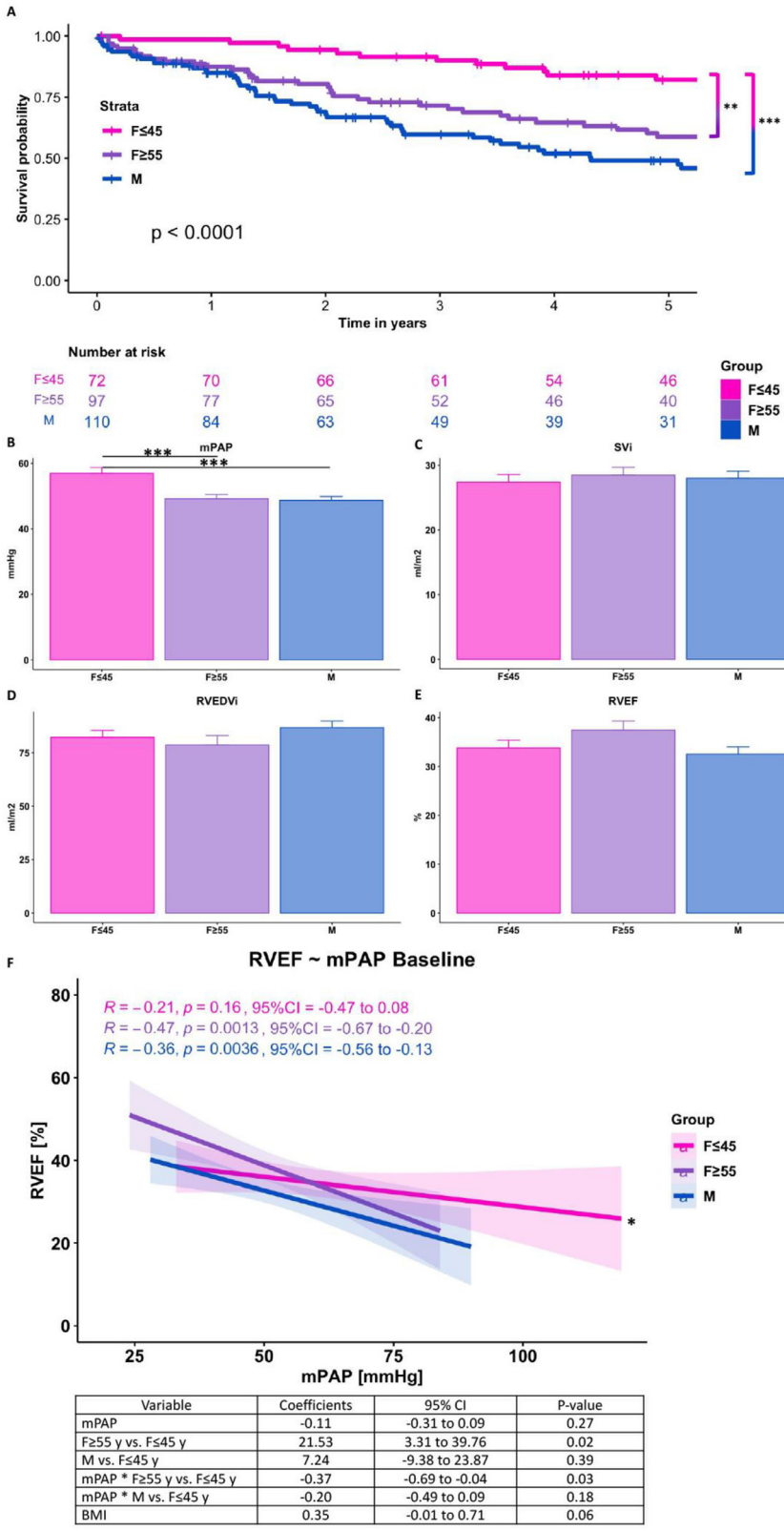
[b]Females ≤45 vs males

[c]Females≥55 vs males.

(EDIPHY trial: NCT03648385). Interestingly, in our cohort the relation of DHEA-S with RV dysfunction was more pronounced in male PAH-patients. Therefore, sex-specific analyses of treatment effects of DHEA-S supplementation are of interest.

Possible detrimental relation between androgens and RV function

Androgens may also be involved in RV adaptation to pressure-overload. Elevated levels of androgens are



Variable	Coefficients	95% CI	P-value
mPAP	-0.11	-0.31 to 0.09	0.27
F≥55 y vs. F≤45 y	21.53	3.31 to 39.76	0.02
M vs. F≤45 y	7.24	-9.38 to 23.87	0.39
mPAP * F≥55 y vs. F≤45 y	-0.37	-0.69 to -0.04	0.03
mPAP * M vs. F≤45 y	-0.20	-0.49 to 0.09	0.18
BMI	0.35	-0.01 to 0.71	0.06

Figure 4 **Five-year survival of PAH-patients and hemodynamics and RV phenotype at baseline.** Females are stratified on age into females of reproductive age (≤45 years) and females of postmenopausal age (≥55 years) to get an idea of the contribution of estrogens to RV adaptation. Sex and age differences in the 5-year transplant-free survival probability in male (N = 110) and female PAH-patients of reproductive age (≤45 years, N = 72) and females of postmenopausal age (≥55 years, N = 97) in (A) Baseline measurements of mPAP in (B) SVi (C) RVEDVi (D) and RVEF (E) and the relation between mPAP and RVEF as measure for RV adaptation (F) in PAH females ≤45 years compared to females ≥55 years and males. Differences between groups were tested using interaction testing as depicted by the interaction term in the table. ** $p < 0.01$; *** $p < 0.001$. F ≤45, Females ≤45 years; F ≥55 = Females ≥55 years; M = Males; mPAP, mean Pulmonary Arterial Pressure; RVEF, Right Ventricular Ejection Fraction; RVEDVi, Right Ventricular End-Diastolic Volume index; Svi, Stroke Volume index.

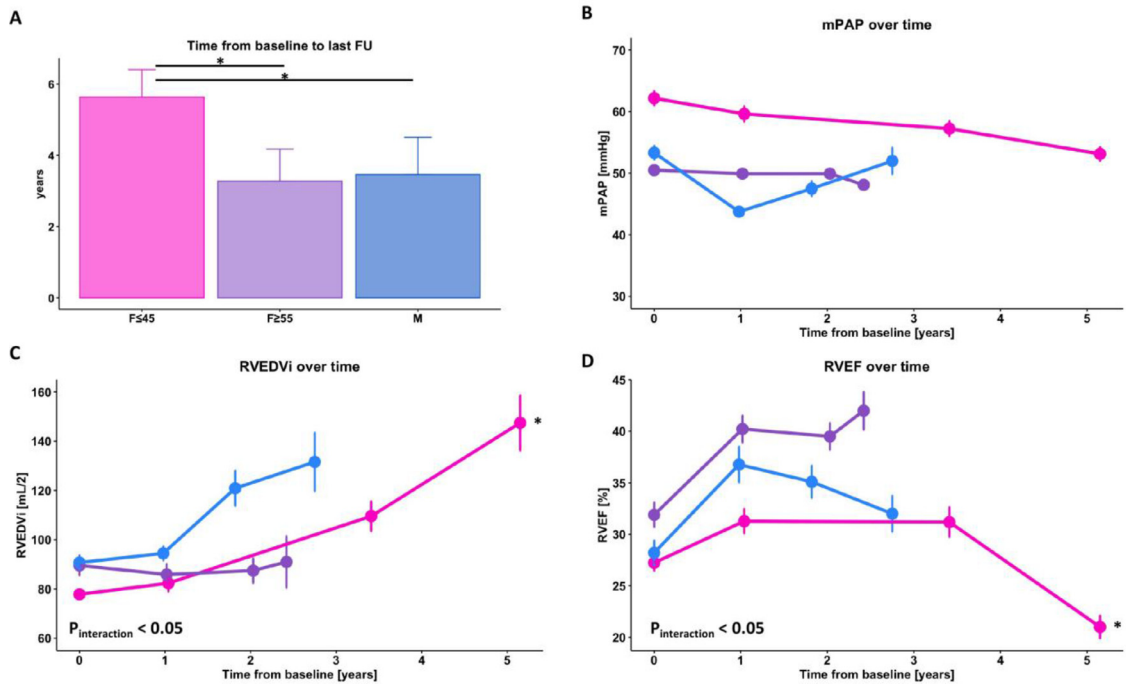


Figure 5 Change in hemodynamics, RV morphology and function over time from baseline to last follow-up before an event. Time from baseline to last follow-up measurement before an event (death or transplantation) in (A) Change in measurements from baseline over time for mPAP (B) RVEDVi (C) and RVEF (D) to last follow-up with a maximum of 1.5 years before an event in PAH females of reproductive age (≤45 years, N = 19) compared to females of postmenopausal age (≥55 years, N = 15) and to males (N = 15). Differences between and within groups over time were assessed with a repeated measures ANOVA after which post-hoc testing with t-tests and Bonferroni correction were applied. $P_{interaction}$ represents the interaction between group and time point. * indicates $p < 0.05$ for two post-hoc pairwise tests at last follow-up in F ≤45 vs F ≥55 and vs M. FU, Follow-up; F ≤45, Females ≤45 years; F ≥55, Females ≥55 years; M, Males; mPAP, mean Pulmonary Arterial Pressure; RVEDVi, Right Ventricular End-Diastolic Volume index; RVEF, Right Ventricular Ejection Fraction.

associated with higher RV mass and volumes in males.[11] Interestingly, we show for the first time that plasma testosterone is differentially associated to RV dysfunction in male and female PAH-patients. Despite a reduction in DHEA-S in both male and female PAH-patients, we only detect lower levels of androstenedione and testosterone in females, not in males.[15] An alternative source of androstenedione production is through conversion of progesterone.[13,29] However, the effect progesterone has been poorly studied in both experimental PAH and patients, and therefore the current knowledge is limited. In contrast to females, the progesterone pathway may be upregulated in male PAH-patients leading to a preservation of testosterone and estradiol levels. Despite preserved levels, testosterone was associated with RV dysfunction in male PAH-patients, suggesting that testosterone levels may negatively affect RV adaptation. This is in line with experimental data in which testosterone was associated with dysfunctional RV hypertrophy and excessive RV fibrosis in male mice with pulmonary artery banding.[30] This suggests that androgens may have a detrimental effect on RV adaptation to pressure-overload, predisposing to an earlier onset of RV failure and possibly resulting in worse survival in males. Hence, a lack of testosterone may be protective in females.

Worse end-stage disease phenotype in PAH females

Female PAH-patients have a better prognosis and, at the same time, the worst RV phenotype at end-stage disease, which can be appreciated by more RV dilation and worse RV function shortly before death or transplantation. Our histopathologic results confirmed more hypertrophy, decreased capillary density and increased apoptosis in PAH RV tissue. Unfortunately, we cannot draw definite conclusions regarding sex differences with this limited number of human end-stage RV samples; thus, novel translational tools, like cardiac tissue engineered models, are highly necessary. Increased estrogen or reduced testosterone levels may protect the female heart and allow better endurance to maintained pressure-overload, subsequently resulting in a worse stage of RV failure at a later point in time. Male patients showed a moderate decline in RV function from baseline compared to females of reproductive age, but at a much earlier point in time. Interestingly, females of postmenopausal showed preserved RV function before an event. Therefore, the remaining question is whether older female die at an earlier disease stage due to other causes than RV failure. Both older female and male patients from our cohort frequently had comorbidities, such as smoking and a

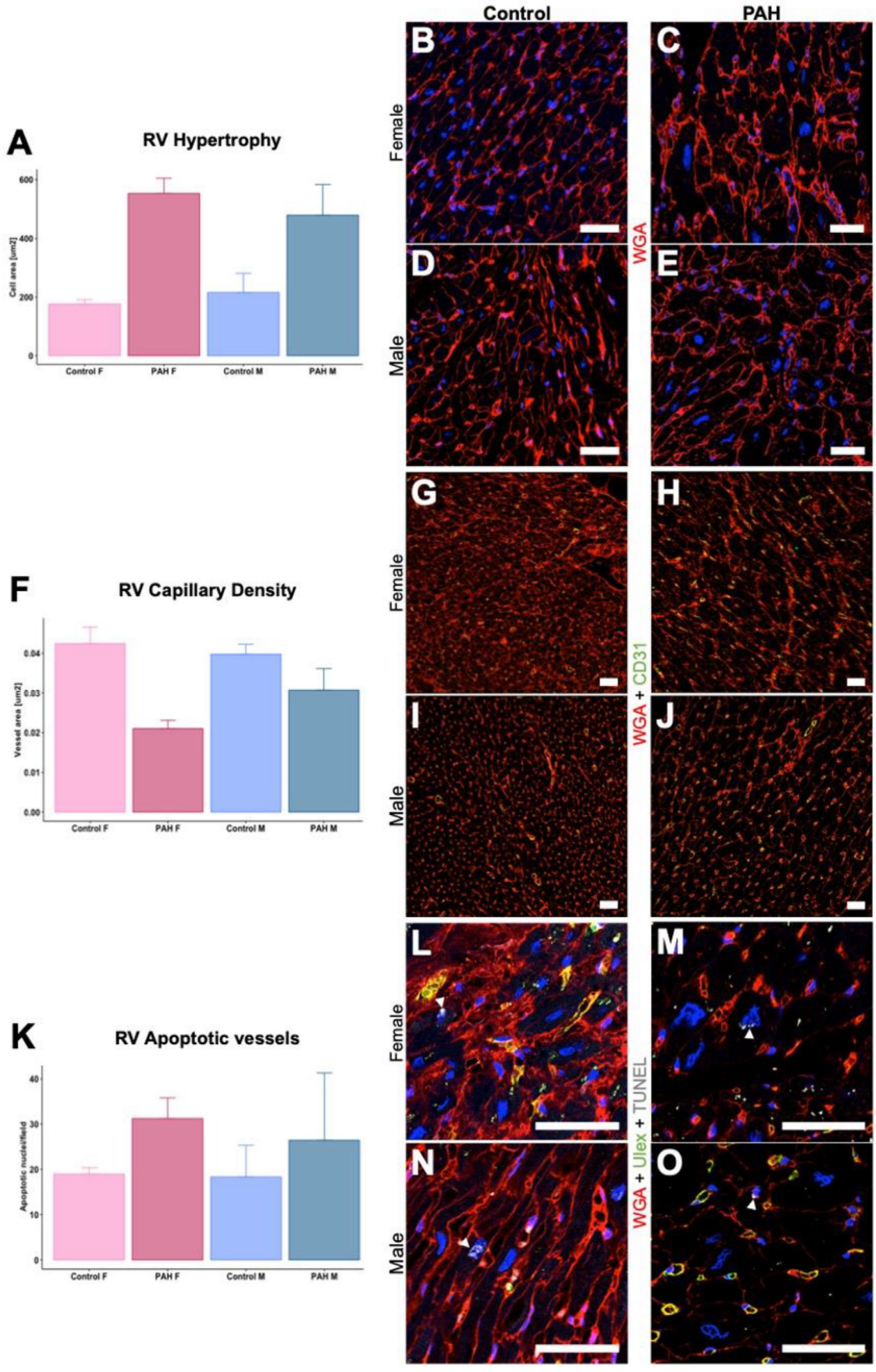


Figure 6 **Histopathological analyses in RV tissue of PAH-patients and controls.** RV cardiomyocyte cross-sectional area quantification (Hypertrophy, A) and representative pictures for cell membrane counterstaining (WGA; B-E). Vascular density quantification (F) and illustrative overviews (CD31, green; G-I). Vascular apoptosis quantification (K) and characteristic stainings (L-O) against apoptotic nuclei

reduced DLCO, previously associated with worse survival in PAH.[31] Unfortunately, as we were unable to retrieve cause of death in patients due to Dutch regulations, we cannot draw definite conclusions which we acknowledge to be a limitation to this study. However, we were able to investigate statistically whether the survival difference could be explained by differences in comorbidities and DLCO. For this purpose, we have repeated the cox regression analyses and included comorbidities and DLCO as covariates. The survival difference between the groups remained significant after correction, indicating that the difference between comorbidities and DLCO could not explain the survival difference observed between males, females≥55years and females≤45years

Limitations and strengths

Although the large sample size of the age-stratified PAH cohort (N = 279) is one of the strengths of this study, one of the limitations of this study is the relatively small sample size of the sex hormone and end-stage disease cohort. The relative small sample size of these cohorts limited the ability to perform extensive multivariate statistical analyses. Especially the male sample size is limited, nonetheless, the smaller representation of males is reflective of the PAH-population. As PAH is a rare disease,[32] the time span of inclusion was wide to include a significant number of patients. This may have led to a potential bias in the PAH-population as treatment strategies have changed over time. However, we corrected the survival analysis for differences in treatment that may arise over time, but differences between groups remained significant. The selection patients in the end-stage disease cohort on an event and the availability of several follow-up timepoint may have led to a bias as well, however, this would be similar in all groups.

Furthermore, there were small differences in disease severity between the baseline cohort and the cross-sectional cohort, which may be explained by differences in treatment. However as the cross-sectional cohort included both treatment naïve and treated patients, it may be the most reflective of the PAH patient population. In addition, we did not have information on the menstrual cycle and use of oral contraceptives (OCP) and HRT at time of blood sampling. However, data on OCP use in the Netherlands is available and HRT use in the Netherlands is low compared to the US.[18,33] In addition, due to the inter- and intra-variability in estrogen levels in females, we did not measure estradiol levels in female PAH-patients, but only in male subjects. Although this is a limitation to the study, the comparison of females ≤45years to females >55 years provides insight into an association between estrogen levels and RV adaptation. Lastly, availability of human end-stage disease RV tissue samples is limited, and therefore numbers for the histopathological analysis were small, and we could not match patient samples on age nor perform statistical analysis. In addition, 8 of 12 RV samples were obtained at the VUmc, whereas the other four were obtained from the French cohort, in which clear sex differences have been previously reported.[6] The histopathological analyses suggest more capillary rarefaction and apoptosis in female patients, providing a hypothesis for future studies. New approaches are necessary to overcome limited RV tissue availability, and to investigate the direct effect of sex hormones on the right ventricle throughout in vitro cardiac models to study the cardiomyocyte/endothelial cell interaction in females. However, even with limited numbers, we have provided insight into underlying pathophysiological mechanisms of RV failure in end-stage disease of PAH-patients. The strength of our study is the combination of RHC and CMR longitudinal data with cross-sectional sex hormone and histopathological analyses of end-stage disease RV tissue. Importantly, we show that previous findings on sex hormone alterations in PAH can be extrapolated to a European cohort of PAH-patients across a wide range of ages. In addition, we used ID-LC-MS/MS for quantification of sex hormone levels, which has shown to be more reliable and accurate compared to immunoassays.[34] Finally, although follow-up is performed on a regular basis in our clinic, additional follow-up may have been indicated in patients with clinical deterioration, which could have led to a bias in the follow-up population. However, this would be the case in all groups and would not explain differences.

Conclusions

In a large Dutch cohort of PAH-patients across a wide range of ages, we show for the first time a differential association between sex hormones and RV function in male and female PAH-patients. While DHEA-S levels appeared lower in both male and female patients, androstenedione and testosterone levels tended to be reduced in females only. Our longitudinal data suggest that females of reproductive age may persevere pressure-overload for a longer time period resulting in a worse RV phenotype at end-stage disease.

Disclosure statement

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(TUNEL, gray) and vessels (Ulex, green). White arrowheads indicate apoptotic cells. Nuclei were counterstained with Hoechst 33342 (blue, B-E and L-O) and WGA (red). N = 3 subjects/group and ≥10 fields/subject. Scale bars = 50μm. CD31, Cluster of Differentiation 31; PAH, Pulmonary Arterial Hypertension; RV, Right Ventricular; Ulex, Ulex europaeus agglutinin; WGA, Wheat Germ Agglutinin.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.healun.2021.11.004.

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